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(54) Process for isolating nucleic acid.

(57) The invention relates to a process, a combination of means for isolating nucleic acid from a nucleic acid-containing starting material and a testkit in order to amplify the nucleic acid obtained by said process. More in particular, the invention relates to a process and a kit for isolating nucleic acid from a nucleic acid-containing biological material such as whole blood, blood serum, urine, feces, cell cultures and the like.

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gram-positive bacteria and some yeasts and moulds, cannot immediately function as an input material in the process according to the present invention, because owing to their special cell wall structure they do not lyse into a chaotropic substance. Therefore, such starting materials require a pretreatment rendering the cells accessible, e.g., a preceding cell lysis, after which the resulting lysate can be subjected to the process according to the invention.

By nucleic acid (NA) is meant both DNA and RNA, both in any possible configuration, i.e. in the form of double-stranded (DS) nucleic acid, or in the form of single-stranded (ss) nucleic acid, or as a combination thereof (in part ds or ss).

Essential according to the invention is the use of a nucleic acid binding solid phase e.g. silica particles capable of binding the NA in the presence of a chaotropic substance. By silica are meant SiO_2 crystals and other forms of silicon oxide, such skeletons of diatoms built up from SiO_2, amorphous silicon oxide and glass powder. Also alkylsilica, aluminium silicate (zeolite), activated silica with $-NH_2$, latex particles, certain polymeric materials forming the inside wall of a cuvette or a microtiter plate, or filter materials for example consisting of nitrocellulose are suitable as nucleic acid binding solid phase according to the invention.

For the matter using silica particles, it was known from PNAS 76, 1979, 615, that dsDNA in a highly concentrated solution of chaotropic salt NaI (sodium iodide) can be released from agarose and can be bound to glass. This publication describes two procedures for isolating DNA from an agarose gel, both of which use in a first step an NaI solution to dissolve the agarose. In one procedure the DNA is precipitated in a second step with acetone, while according to the other procedure the DNA is bound in a second step to glass particles and is then eluted into an aqueous buffer. This method, however, is of no use to more complex starting materials, such as body fluids and other biological starting materials. In this article there is also no disclosure for an one-step procedure according to the invention.

It is recommendable according to the invention to use silica particles having a suitably selected particle size so that a high degree of purity of the bound and then eluted nucleic acid is immediately obtained from an impure starting material.

A preferred embodiment of the invention is characterized by using silica particles having a practical size ranging substantially between 0.05 and 500 µm. By the term "substantially" is meant that 80% or more, preferably more than 90%, of the silica particles are within the particle size range defined. In order to ensure easy processing of the bound NA, it is preferred that the silica particles employed have a particle size range substantially between 0.1 and 200 µm, while a process in which the silica particles employed have a particle size ranging substantially between 1 and 200 µm is most preferred. It is true that the NA-binding capacity of the silica particles is higher as the particles are smaller, but especially in the case of an NA-rich input material and in the case of relatively long NA molecules the use of extremely small silica particles will result in that the NA-silica complexes formed cannot be efficiently redispersed anymore. This means that the bound NA cannot be recovered from the complexes in a pure form. When human blood is used as an input material, this problem sometimes occurs if there is used a non-fractionated silica having particle sizes within the range of 0.2-10 µm. The formation of aggregates that cannot be redispersed anymore may be avoided by using a fractionated silica, the particle size of which is within the range of 1-10 µm. When an input material rich in cells is used, such as bacterial cultures, it is found, however, that the use of such a coarse silica fraction is not sufficient to avoid the formation of hardly redispersible aggregates and optimum results are obtained if there is used an even coarser silica, such as a diatomaceous earth having particle sizes within the range of 2-200 µm.

In another preferred embodiment the NA binding solid phase is in the form of a filter or even forms part of the vessel in which the sample with chaotropic substance is contained. The latter forms for the NA binding solid phase eliminates the necessity of centrifugation or filtration for further sample processing and NA isolation.

According to the invention it is essential to use a chaotropic substance in addition to the above-mentioned nucleic acid binding solid phase such as silica particles. By chaotropic substance is meant any substance capable of altering the secondary, tertiary and/or quaternary structure of proteins and nucleic acids, but leaving at least the primary structure intact. Examples thereof are guanidinium (iso)thiocyanate and guanidine hydrochloride. Also sodium iodide, potassium iodide, sodium (iso)thiocyanate, urea or mutual combinations therewith are very suitable in combination with nucleic acid binding solid phases for the isolation of NA from a nucleic acid-containing starting material. According to the invention the chaotropic guanidinium salt employed is preferably guanidinium thiocyanate (GuSCN).

The process according to the invention will usually be carried out in such a way that the starting material is mixed with sufficiently large amounts of chaotropic substance for instance guanidinium salt and for instance silica particles to release essentially all of the nucleic acid present in the starting material and bind it to said silica particles. A suitable protocol is, e.g., the addition of a suspension of silica particles to a

MATERIALS AND METHODS

A) Suspension of Silica Coarse (SC)

Use was made of silicon dioxide (SiO_2), supplied by Sigma, having a particle size distribution of 0.5-10 µm, 80% of which ranged between 1 and 5 µm.

60 g silica were suspended in aqua bidest (up to a volume of 500 ml) in a cylinder having a diameter of 5 cm; the height of the aqueous column was 27.5 cm. After 1x g sedimentation for 25 hrs at room temperature supernatant was sucked off, until 70 ml were left. Aqua bidest was added up to 500 ml, and the particles were resuspended by shaking the cylinder. After 1x g sedimentation for 5 hrs supernatant was sucked off, until 60 ml were left. After addition of 600 µl 32% (w/v) HCl the particles were resuspended by vortexing. The suspension was made up in aliquots of 4 ml in 6 ml bottles, which were tightly closed and heated in an autoclave at 121 °C for 20 min. This sedimentation protocol led to an enrichment of the larger silica particles, i.e. the particles having a particle size above 1 µm, as was established by an electron-microscopic examination. Moreover, the autoclave treatment of an acid (pH about 2) silica suspension results in that optionally present nucleic acid is fully degraded. The thus obtained suspension of Silica Coarse will hereinbelow be referred to as SC.

Suspensions of Silica derivatives

Silica was derivatized with methylacrylamide silicondioxide having alkyl-tails with a length of 2 to 18 C-atoms. The size of the derivatized silica particles varied from 63 to 200 µM. The pore size of the particles used was 500 Å. These silica derivates (12 $MAAMC_2$-C_{18}) were supplied by Diosynth, Oss.

For the NA isolation (example H1) 0.5 g of the derivatized silica particles were suspended in 1 ml aqua bidest. These silica suspensions were pretreated with 120 µl 32% (w/v) HCl for 30 min. at 90 °C.

Suspensions of polystyrene latex particles

Use was made of two types of polystyrene latex particles. The polystyrene latex VQ69 red had been absorbed with sodium-dodecylsuccinate sulfate groups and has a particle size of 424 nm. The polystyrene latex VQ58B had a smaller size (328 nm) and no sulfate group has been absorbed on the outside.

Use was made of three, hydrophilic, glycidylmethacrylaat polystyrene latex particles. The size of AGF27G; ACN3 red and AGY1515 were 933 nm, 206 nm and 846 nm respectively. All the mentioned polystyrene particles were supplied by ARLA-Arnhem.

Commercial filters

Use was made of

1. PVDF an Immobilon Transfer Membrane (hydrophobic) supplied by Millipore.
2. Nitrocellulose supplied by Schleicher and Schuell (0.2 µM Ref.no.401.396).
3. Hybond-N a Nylon Hybridization membrane (0,45 micron, lot: 16872) supplied by Amersham.

B) L2 buffer

L2 buffer (0,1 M Tris.Cl pH 6.4) was prepared by dissolving 12.1 g TRIS (Boehringer) in 800 ml aqua bidest, adding 8.1 ml 37% (w/v) HCl and bringing the volume to 1 litre with aqua bidest.

C) Washing liquid L2

The washing liquid L2 was prepared by dissolving 120 g GuSCN (guanidine thiocyanate of Fluka) in 100 ml L2 buffer.

I) Washing procedure

A pellet is washed by adding 1 ml washing liquid, then vortexing until the pellet is resuspended, centrifuging for 15 sec. at 1200x g, and discarding the supernatant by suction.

J) Elution procedure

The elution takes place by adding at least 25 µl, preferably at least 40 µl elution buffer, vortexing briefly (2 sec) and incubating for 10 min. at 56 °C.

K) Protocol B

This protocol is suitable for isolating dsDNA from complex starting materials, such as human serum, whole blood, watery feces or urine and makes use of Eppendorff test tubes with 900 µl GEDTA and 40 µl SC.

1. Vortex test tube until pellet is resuspended
2. Add 50 µl starting material (e.g., serum, whole blood, feces or urine) and vortex immediately until homogeneous (5-10 sec.).
3. Leave at room temperature for 10 min. and vortex 5 sec.
4. Centrifuge for 15 sec. at 12000x g and discard supernatant by suction.
5. Wash pellet once with GEDTA.
6. Wash pellet twice with 70% ethanol.
7. Wash pellet once with acetone.
8. Dry pellet for 10 min. at 56 °C with open lid.
9. Elute DNA with 50 µl TE buffer without RNAsin.
10. Centrifuge for 2 min. at 12000x g; supernatant contains DNA.

L. Protocol Y

This protocol is suitable for isolating NA (simultaneous purification of dsDNA, ssDNA, dsRNA and ssRNA) from complex starting materials, such as human serum, whole blood, watery feces or urine and makes use of Eppendorff test tubes with 900 µl L6 and 40 µl SC.

1. vortex test tube until pellet is resuspended.
2. Add 50 µl starting material (serum, whole blood, feces or urine) and vortex immediately until homogeneous (about 5 sec.).
3. Leave at room temperature for 10 min. and vortex 5 sec.
4. Centrifuge for 15 sec. at 12000x g and discard supernatant by suction.
5. Wash pellet twice with L2.
6. Wash pellet twice with 70% ethanol.
7. Wash pellet once with acetone.
8. Dry pellet for 10 min. at 56 °C with open lid.
9. Elute NA with 50 µl TE buffer, optionally in the presence of RNAsin.
10. Centrifuge for 2 min. at 12000x g; supernatant contains NA.

Protocol Y*

This protocol is suitable for isolating NA from complex starting materials, such as human serum, urine or bacterial cultures.

Procedure:

Eppendorff tubes were used with 900 µl L6* and 40 µl SC.

1. Vortex test tube until pellet is resuspended.
2. Add 50 µl starting material (serum-plasmid, urine-plasmid mixtures or overnight bacterial culture) and vortex immediately until homogeneous (5 sec.).
3. Leave at roomtemperature for 10 min. while mixing.

DNA

This section E comprises experiments showing that the invention can be used for isolating ssDNA.
section F: diatomaceous earth

This section F shows that diatom skeletons are very useful as the silica particles to be used according to the invention. It is also shown that the invention can be used for isolating NA from different gram-negative bacteria.

Section G shows that NA can be purified from bacterial cells using various chaotropic substances.

Section H and I show the isolation of DNA with the aid of alternative solid phases.

There was always used an amount of 50 µl. The blood used in section B and F was always fresh blood drawn off in the presence of EDTA to prevent clotting (using the Venoject system of Terumo N.V., Louvain, Belgium, collecting tubes of the type VT-574 TKZ). The starting materials used in the other sections (serum, urine and feces) were frozen. In examples A1, A2, A3, B1, B2, B5, B7 and F1 the serum or blood was from the same subject.

O) Further methods

For gel-electrophoretic examination, part of the eluted amount of NA was loaded on a neutral agarose-gel containing 1 µg/ml ethidium bromide in the buffer system described by Aaij and Borst (Biochim.Biophys.Acta 269, 1972, 192). Photographs were taken under UV illumination of the gel.

In some experiments a known amount of a purified DNA (input DNA) was added to the clinical sample. In these cases an amount of input DNA corresponding to an extraction efficiency of 100% was also loaded on the same gel.

Bacterial plasmid DNA was purified as described by Ish-Horowicz and Burke (Nucleic Acids Res. 9, 1981, 2989) from Escherichia Coli HB101, followed by column chromatography with Sepharose CL 2B (Pharmacia, Inc.) and ethanol precipitation. Bacterial plasmid DNA was purified from Escherichia Coli JM101 (J. Messing, Rec. DNA Techn. Bull. 2:43-48(1979) as described by Birnboim and Doly (Maniatis, T. et al., Molecular Cloning, CSH, New York). The pCMV-E contains a 0.4 kb human cytomegalo virus DNA fragment cloned in the 2 kb vector pHC 624 (Boros in Gene 30, 1984, 257); pEBV-10 contains a 0.9 kb Epstein Barr virus DNA fragment cloned in the same vector. To obtain a plasmid preparation enriched for relaxed circular (CII) molecules, pEBV-10 DNA (2.9 kb) was treated with DNAse I. Component II molecules serve as a model for purification of Hepatitis B viral DNA which is present in virions as a 3.2 kb relaxed circular DNA molecule.

The pGem3p24 contains a 1,45 kb HIV sequence; the construction of pGem3p24 is described below.

The sequence of HIV HxB2 DNA has been described by several authors (J. Virol. 61, 633-637(1987); Nature 326, 711-713(1987); Aids Res. Hum. Retrovirus 3, 41-55(1987); Aids Res. Hum. Retrovirus 3, 33-39-(1987) and Science 237, 888-893(1987)).

HIV HxB2 DNA was partially cleaved with FokI at sites 1189 and 2613 of the original HIV HxB2 sequence. The nucleotide nrs. refer to the Genebank designation.

The FokI sites of this fragment were filled up using Klenow DNA polymerase (Maniatis, vide supra) and cloned (Maniatis, vide supra) in the polylinker SmaI-site of plasmid pUC-19. The resulting plasmid which carries the HIV HxB2 DNA fragment was called pUC19-p24.

To obtain plasmid pGem3p24, the 1450 bp EcoRI-BamHI fragment of pUC19-p24 was cloned in the EcoRI-BamHI digested vector pGem3 (2867 bp; Promega Corporation, Madison USA).

The primers used in the PCR method were synthesized on an oligo-synthesizer apparatus (from Applied Biosystem). Nucleotide sequence of the primers ES47 (25 mer) and ES75 (47 mer) are given below.

Example A1: DNA purification from human serum

Human serum (500 µl) was mixed with known amounts of purified DNA [100 µl LMW (45 µg), 20 µl MMW (20 µg), 40 µl CI/II (20 µg)] and 10 samples of 66 µl were used as input material for 10 DNA extractions according to protocol B. The amount of SC (suspension of Silica Coarse) present in the test tubes was varied in this experiment between 2.5 and 40 µl. The extractions were carried out in duplicate and half (30 µl) of the eluted DNA from each sample was electrophoresed through a 1% agarose gel. For comparison, half of the amount of input DNAs were also loaded on the same gel in control lanes.

Double-stranded DNA, both linear (range 23 kb to approximately 60 bp), covalently closed (CI) and relaxed circular (CII) DNA were efficiently isolated if the amount of SC exceed 10 µl. The yield of the largest MMW fragment (approx. 23 kb) seems relatively low when compared to the smaller fragments, which in view of other experiments, may be attributed to shearing of high molecular weight fragments.

The control lanes show respectively the amount of LMW, CII/CI and MMW DNA which would be found in an extraction efficiency of 100%. As previously stated, a CII-rich (DNAse I-treated) 3 kb plasmid (pEBV-10) was used as input material.

Example A2: DNA isolated from human serum is a good substrate for restriction enzymes and T4 DNA ligase

Purified DNA preparations were added to 12 human serum samples of 50 µl. DNA was isolated from these 12 mixtures according to protocol B; elution was effected with 50 µl TE. Half of the eluted DNA was treated (in duplicate) either with one of the following three restriction enzymes: EcoRI, BamHI and BglII (these are active in low-salt, medium-salt and high-salt buffers, respectively), or treated with T4 DNA ligase, or not treated. The DNA samples were electrophoresed through a 1% agarose gel and visualized by UV illumination.

The results of the T4 ligase treatment (1 h at 37 °C, 3 units of T4 ligase in a 30 µl reaction volume) shows a shift in molecular weight of the DNA fragments and indicates that the DNA isolated from human serum is not significantly affected by exonucleolytic degradation.

The results for 8 serum samples to which a purified plasmid (pCMV-E; 3.3 µg; 1.5 µl) was added shows respectively that for EcoRI, BamHI and BglII digests all restriction enzymes linearized the plasmid. All restriction enzyme incubations were done in a 30 µl reaction volume for 1 h at 37 °C with 9 units of enzyme.

Example A3: simultaneous isolation of DNA and ssRNA from a human serum

Since in human serum only very low levels of RNA are present (e.g., in viruses, bacteria or cells) which are not detectable by UV illumination of ethidium-bromide stained gels, exogenous RNA sources were added to human serum samples. Mammalian cells or bacteria were used as exogeneous RNA sources. The NA was isolated from the samples according to protocol Y and eluted in 50 µl TE with 0.5 U RNAsin per µl in the absence or in the presence of RNAseA (40 ng per µl of the elution buffer). The results of the subsequent electrophoresis through a 1% agarose gel shows that RNA and DNA can be detected. The mammalian cells added were per 50 µl serum sample $5x10^5$ rat 10B cells (Boom et al., J. Gen. Virol. 69, 1988, 1179) while the bacteria added were per 50 µl serum the cell pellet of a 100 µl overnight culture of the E. coli strain HB101 containing the plasmid pCMV-E.

Example A4: Polymerase chain reaction for the detection of Human Immunodeficiency Virus RNA isolated from human serum

NA (75 µl) was isolated from 2 human serum samples of 50 µl each (patients F and H) according to protocol Y. The serum of patient F contained a high (2700 pg/ml) level of the HIV antigen P24 (according to the HIV P24 antigen solid phase immunoassay of Abbott Laboratories) but was negative for HIV antibodies (according to the HIV antibodies ELISA of Abbott Laboratories), and the serum of patient H was negative in both tests.

Part of the isolated NA (43 µl) was treated with RNAse-free DNAse (Boehringer; 1 U DNAse/µl) for 90 min at 37 °C. After ethanol precipitation and heat inactivation for 15 min at 68 °C, the RNA was suspended

One ml of human blood contains approx. $5x10^9$ erythrocytes which are non-nucleated and do therefore not contribute to the NA amount of blood. The NA amount of blood is largely determined by the white blood cells (approx. $4\text{-}10x10^6$ per ml). These cells are embedded in an aqueous medium (plasma) containing large amounts of proteins (approx. 70 mg/ml blood). Thus, whole blood is an extremely unpure source for NA purification. The examples of section B show that notwithstanding NA can be isolated from whole blood by protocols B and Y.

Example B1: DNA isolation from human whole blood

Human blood (500 µl) was mixed with known amounts of purified DNA 100µl LMW (45 µg), 80 µl CI/II (40 µg) and 10 samples of 68 µl were used as input material for 10 DNA extractions according to protocol B. In this experiment the amount of SC (suspension of Silica Coarse) present in the test tubes was varied between 2.5 and 40 µl. The extractions were carried out in duplicate and half (30 µl) of the eluted DNA from each sample was electrophoresed through a 1% agarose gel. For comparison, half of the amount of input DNAs was also loaded on the same gel.

Double-stranded DNA, both linear, covalently closed (CI) and relaxed circular (CII) DNA, was efficiently isolated from human whole blood if more than 10 µl SC, were used. The amount of DNA recovered from whole blood was proportional to the amount of SC up to approximately 10 µl. Higher amounts seemed to be saturating.

Example B2: DNA isolated from human whole blood is a good substrate for restriction enzymes and T4 DNA ligase

Purified DNA preparations were added to 12 human blood samples of 50 µl. The DNA was isolated from these 12 mixtures according to protocol B; elution occurred with 50 µl TE. Half of the eluted DNA was either treated with one of the following three restriction enzymes: EcoRI, BamHI and BglII (these are active in low-salt, medium-salt and high-salt buffers, respectively), or treated with T4 DNA ligase, or not treated. The DNA samples were electrophoresed through a 1% agarose gel and visualized by UV illumination.

The results of T4 ligase treatment (1 h at 37 °C, 3 units of T4 ligase in a 30 µl reaction volume) shows a shift to a higher molecular weight of the DNA fragments and indicates that the DNA isolated from human blood is not significantly affected by exonucleolytic degradation.

The results for 8 blood samples to which a purified plasmid (pCMV-E; 3.3 µg; 1.5 µl) was added show that for EcoRI, BamHI and BglII digests all restriction enzymes linearized the plasmid. All restriction enzyme incubations were done in a 30 µl reaction volume for 1 h at 37 °C with 9 units of enzyme.

Example B3: DNA isolation from 10 different samples of blood

In this example 10 different samples of human blood randomly chosen from a blood bank are used as starting material. Of each of the samples the number of white blood cells (WBC) was known. DNA was purified from 50 µl of the samples according to protocol B, and elution occurred with 75 µl TE. One third of the isolated DNA was directly applied to a 1% agarose gel and part (2 µl) of the remainder was used for a PCR.

The same samples were subjected to the same isolation procedure after 3 µl LMW-DNA (6 µg) was added to each 50 µl sample. Here, too, 25 µl of the eluate (75 µl) was directly applied to the gel; another portion of 25 µl of the eluate was first treated with T4 DNA ligase (1 h at 37 °C, 2 U in a reaction volume of 30 µl) and then applied to the same gel.

The content of white blood cells (WBC) of blood samples 1-10 was as follows:

quently isolated according to protocol Y (example C2).

Example C3 shows that DNA can be purified from human urine with alternative chaotropic substances such as KI, NaI and NaSCN instead of GuSCN with silica as nucleic acid binding solid phase according to protocol Y*.

Example C1: DNA purification from human urine

3 µl LMW DNA (6 µg) was added to 10 randomly chosen human urine samples of 50 µl with varying turbidity (samples 4, 5, 6 and 7 were clear, samples 1, 2, 3 and 8 were slightly turbid, and samples 9 and 10 were very turbid). The DNA was isolated according to protocol B and eluted with 75 µl TE buffer. One third of each eluate was applied to a 1% agarose gel. Another part of 25 µl was treated with a 1.8 U T4 DNA ligase (1 h at 37 °C in a 30 µl reaction volume) and applied to the same gel. Marker lanes contain respectively LMW DNA and MMW DNA. The amount of LMW DNA (2 µg) in a marker lane represents the amount to be observed with an extraction efficiency of 100%.

The results show that DNA can be efficiently purified from human urine with protocol B and is a good substrate for T4 DNA ligase.

The LMW DNA isolated from urine sample No. 10 has been clearly degraded. It was to be expected, however, that naked DNA (as used in this experiment) would be degraded if a urine sample is rich in nucleases. Degradation is therefore likely to have taken place previously during the preparation of the urine/DNA mixtures rather than during purification. The next example (C2) shows that DNA and even ssRNA present in cells (as opposed to naked) can be efficiently recovered from urine sample No. 10.

Example C2: simultaneous purification of DNA and ssRNA from human urine

In this experiment the same 10 urine samples as used in example C1 were mixed with bacteria carrying a 2.4 kb plasmid (pCMV-E). The NA was isolated from these mixtures according to protocol Y and eluted in 75 µl TE buffer with 0.5 U/µl RNAsin. One third of the eluate was electrophoresed through a 1% agarose gel. Another 25 µl portion of the eluate was treated with 10 U of the restriction enzyme EcoRI which linearizes pCMV-E (1 h at 37 °C in a 30 µl reaction volume). This treatment was conducted in the presence of 40 ng/µl RNAseA. The electrophoresis result shows the 23S and 16S ribosomal RNAas as well as the covalently closed (CI) and linear (CIII) forms of plasmid DNA.

Example C3: DNA purification with other chaotropic substances

Human urine (50 µl) was mixed with 400 µl chaotropic substance, lysis buffer L6* and 1 µg pGem3p24 DNA. This total suspension was mixed and added to 500 µl chaotropic substance (see table C3.1) and 40 µl SiO_2 for the purification of DNA according to protocol Y*. The quantity of DNA isolated from urine was analysed using agarose gel electrophoresis. Efficiency of DNA recovery was judged as described in Example A5 and the results are summerized in Table C3.1.

Table C3.1

Recovery of plasmid DNA from human urine samples using various chaotropic substances in combination with Silica (see also legends of Table A5.1)

Sample nr	chaotropic substance used	recovery of pGem3p24 CII	recovery of pGem3p24 CI
1	GuSCN/SiO_2	+	+
2	KI 3M/SiO_2	+	+
3	NaI 3M/SiO_2	+	+
4	NaSCN 3M/SiO_2	+	+

material for NA extractions according to protocol Y, and 40 µl SC or 40 µl of the Celite 521 suspension were used as NA carrier.

The extractions in which SC was used had to be stopped during the first wash since the NA silica complexes could no longer be homogenized, not even after vortexing for a long time (over 3 min.). On the other hand, extractions in which Celite 521 was used proceeded without problems, presumably due to the larger particle sizes of the diatomaceous earth relative to the SC particles. The NA was eluted with 70 µl TE buffer without RNAsin and part of the eluate (20 µl) was electrophoresed through a 1% agarose gel.

The marker lanes contain 1 µg MMW DNA. Results for the following types of bacteria were obtained:

1 : Campylobacter pylori
2 : Yersinia enterolytica type 3
3 : Neisseria meningitidis
4 : Neisseria gonorrhoeae
5 : Haemophilus influenzae type b
6 : Kelbsiella pneumoniae
7 : Salmonella typhimurium
8 : Pseudomonas aeruginosa
9 : Escherichia coli K1-083

HMW bacterial DNA and rRNAs could be detected using this procedure.

Section G: DNA/RNA purification of Escherichia coli JM101

Isolation of NA from gram negative bacteria is possible according to this invention. In bacterial cells high levels of high molecular weight DNA (HMW DNA) and ribosomal RNA are present. Example G1 shows that NA can be purified from bacterial cells using various chaotropic substances with silica as NA binding solid phase.

Example G1: NA isolation/purification (endogeneous) from bacterial cells with various chaotropic substances and silica as NA binding solid phase

NA was isolated from 50 µl overnight bacterial culture JM101 in presence of 900 µl chaotropic substance and 40 µl SiO_2. The high level of HMW-DNA and endogeneous ribosomal RNA (16S and 23S) allows detection of isolated NA by UV illumination of ethidium bromide stained gels. Isolations were carried out according to protocol Y*, and 25% of the eluted NA (40 µl portions) was analysed on agarose gel.

Table G1:

Relative efficiency of HMW DNA and rRNA isolation from bacterial cell samples using various chaotropic substances in combination with silica

Sample nr.	chaotropic substance used	relative efficiency of HMW-DNA recovery	relative efficiency of rRNA recovery
1	3M KI	1	>1
2	3M NaI	1	1
3	3M NaSCN	1	1

Legend:

Table G1 summarizes the results of the agarose gel analysis. Quantification of HMW-DNA and rRNA recovery has been compared with the procedure where GuSCN was used as chaotropic substance in combination with silica: 1 in table G1 indicates equally efficient DNA or RNA recovery. >1 in table G1 indicates better recovery.

Legend:

The results are summerized in table H1. The expected 290bp HIV amplimer fragment was observed in all cases after 30 cycles. The size of the fragments was compared with marker ϕx 174 RF DNA Hae III digest (Pharmacia) also loaded on the gel.

+ + : indicates the detection of the HIV specific 290 bp fragment on the agarose gel at an equal level as using Silica Coarse as solid phase (control).

+ : indicates a detectable level of the 290 bp fragment, lower than the control Silica Coarse.

Section I: Purification with NA-binding filters and GuSCN

NA-binding filters (see Materials & Methods) can replace the SiO_2 in the isolation of nucleic acid according to protocol Y™.

Although normally no release of DNA takes place in the low salt buffer (Tris 10 mM-EDTA 1mM pH 8.0) this optional problem is set aside by inserting the filter with DNA bound to it in the PCR-reaction mixture instead of eluting the DNA from the filter. Example I1 shows that purification of NA can be performed with a NA-binding filters and GuSCN as a chaotropic substance analysed by the PCR-method.

Example I1: DNA isolation/purification with a DNA-binding filter and detection by the PCR-amplification

Pure pGem3p24 DNA (concentration 1 µg; 0,01 µg and 0,005 µg) in 50 µl Tris 10mM/EDTA 1 mM pH 8.0 was added to three DNA-binding filters, (PVDF, Hybond N and Nitrocellulose) with a size of 1 cm x 1 cm and 900 µl GuSCN (lysisbuffer L6).

After washing (no centrifugation steps) and drying at 56 °C (according to protocol Y™) the filter with DNA bound to it was brought directly in the PCR-mixture. In presence of HIV specific primers amplification was performed in the PCR-cycler.

The reaction mixture futher consists of 5 µl 10 x concentrated PCR-buffer, 1 µl 10mM dNTPs, 2 units Taq DNA polymerase and water to a final volume of 50 µl. Subsequently the amplificaton reaction was started.

10 µl aliquots were taken from the raction mixtures after 30 cycles (see example H1) and analysed on a 2% agarose gel.

Table I1:

Detection of DNA isolated using filters as alternative NA binding solid phase in combination with GuSCN as chaotropic substance using PCR-amplification and gel analysis for detection.

sample nr.	NA binding solid phase	Amount of input DNA	Amount of HIVp24 DNA after amplification
1	Hybond N	1.0 µg	+
2	Hybond N	0.01 µg	o
3	Hybond N	0.005 µg	o
4	Nitrocellulose	1.0 µg	+
5	Nitrocellulose	0.01 µg	o
6	Nitrocellulose	0.005 µg	o
7	PVDF-millipore	1.0 µg	+ +
8	PVDF-millipore	0.01 µg	+
9	PVDF-millipore	0.005 µg	+

The result summarized in table I1. The expected 290 bp HIV amplimer fragment was observed. The fragment was compared with a commercial ϕxHaeII

+ +: strong Ethidium bromide stained 290 bp fragment detectable on agarose gel

+: detectable 290 bp fragment

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(54) Process for isolating nucleic acid.

(57) The invention relates to a process, a combination of means for isolating nucleic acid from a nucleic acid-containing starting material and a testkit in order to amplify the nucleic acid obtained by said process. More in particular, the invention relates to a process and a kit for isolating nucleic acid from a nucleic acid-containing biological material such as whole blood, blood serum, urine, feces, cell cultures and the like.

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